MICROBIAL SYMBIONTS OF GREAT BARRIER REEF SPONGES

ADAM M. BURJA, NICOLE S. WEBSTER, PETER T. MURPHY AND RUSSELL T. HILL

Burja, A.M., Webster, N.S., Murphy, P.T. & Hill, R.T. 1999 06 30: Microbial Symbionts of Great Barrier Reef Sponges. *Memoirs of the Queensland Museum* **44**: 63-75. Brisbane. ISSN 0079-8835.

Microbial symbionts of two sponges, *Rhopaloeides odorabile* (Dictyoceratida: Spongiidae) and a new species 'Very White Fan' (VWF) (Dictyoceratida: Phyllospongiidae), are being studied in detail. Bacteria isolated from *R. odorabile*, VWF, and the surrounding ambient seawater were characterised using morphological, biochemical, and molecular techniques. In the case of *R. odorabile*, a single bacterium, designated NW001, was found to dominate the culturable bacterial community associated with the sponge but was absent from ambient seawater samples. Strain NW001 was predominant in all individual sponges sampled (N=40) from different regions of the Great Barrier Reef, generally at more than an order of magnitude greater than the second most common bacterium (NW002). The bacterial community associated with *R. odorabile* appears to be highly stable. In the case of VWF, the culturable bacterial community was more diverse and showed greater variation between individuals. This community generally comprised eight predominant bacteria, rarely isolated from water samples and constituting ca. 70% of the total culturable bacteria. Extensive biochemical testing was performed on all isolates to give data for cluster analyses to identify the major groups of bacteria present. One isolate from each sponge was characterised at the molecular level by PCR amplification and sequencing of 16S ribosomal RNA gene fragments. Analysis of sequence from NW002 indicates it is a *Pseudoalteromonas* sp. Strain E30004315 from VWF is a microalga, with 16S rRNA sequence from its plastid closely related to that of other plastids of marine eukaryotic algae. This study produced an array of well-characterised microbes for natural products screening, in particular for important compounds known to be produced by these sponges. ❐ *Porifera, sponge, symbiont, Dictyoceratida, Demospongiae, 16S rRNA, Rhopaloeides odorabile, microalgae, Vibrio.*

Adam M. Burja & Peter Murphy, Marine Bioproducts Group, Australian Institute of Marine Science, PMB No. 3, Townsville MC, QLD 4810, Australia; Nicole Webster, Faculty of Health, Life, and Molecular Sciences, James Cook University of North Queensland, Townsville, QLD 4811, Australia; Russell T. Hill (email: hillr@umbi.umd.edu), Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202, USA; 4 April 1999.

Symbiosis is considered a permanent association between organisms of different species. This term is not restricted to mutualistic associations but encompasses all associations, regardless of the type of interaction between the individuals. Mutualistic bacterial-invertebrate symbioses have been reported from many invertebrate taxa. Examples of these include cellulolytic nitrogen fixing bacteria from wood boring bivalves (Shieh & Lin, 1994), methanotrophic bacteria of bivalves (Dubilier et al., 1995) and bacterial symbionts of echinoderms (Burnett & McKenzie, 1997; Kelly & McKenzie, 1995). Although chemoautotrophic symbiosis has received the most attention, there are also many symbioses where the type of interaction between the host and their symbiont remains unknown. Most symbiotic bacteria from sponges have been located within the intercellular matrix and can occupy up to 60% of the sponge volume (Wilkinson, 1978a).

The biology of bacterium-sponge associations has elicited considerable interest from researchers investigating novel chemicals derived from sponges. The term symbiont has been broadly applied and few investigators have explored metabolic relationships and capabilities of the symbiont-host complex. One approach that will contribute to understanding these relationships is to isolate symbiotic bacteria and investigate their metabolic and taxonomic characteristics.

Cosmopolitan microbial symbionts associated with marine sponges include heterotrophic bacteria, cyanobacteria and unicellular algae. Numerous studies have described three general classes of heterotrophic bacterial-sponge associations (Wilkinson, 1978a). 1) Small

populations of cosmopolitan bacteria with a species composition similar to that found in the ambient seawater. These are most likely utilised as a food source by the sponge. 2) Species- specific population inhabiting the mesohyl region, not found in seawater, and most likely comprising true symbionts. 3) Fairly ill-defined, consisting of bacteria located within the sponge cells, also likely to be true symbionts. Phenotypically related bacterial symbionts have been described from taxonomically disparate sponges collected from geographically remote locations. Described sponge symbionts have included members of the genera *Pseudomonas, Alteromonas, Vibrio, Aeromonas, Acinetobacter, Micrococcus* and *Moraxella* (Santavy et al., 1990). It has been determined that facultative anaerobic symbionts metabolise a wide range of compounds and may be important in removing waste products whilst the sponges are not circulating water (Wilkinson, 1978a). It has also been postulated that sticky mucoid colonies may be important contributors to sponge structural rigidity (Wilkinson, 1978c). Other functions that have been suggested for sponge bacterial symbionts include digestion of material not available to the host sponge, direct incorporation of dissolved organic matter from the seawater, and digestion and recycling of insoluble sponge collagen.

Sponge symbionts are of biotechnological interest since bioactive compounds of potential medical importance isolated from sponges may be microbial in origin (Bewley & Faulkner, 1998; Bewley et al., 1996; Stierle et al., 1988). There are several practical advantages in isolation of symbionts which produce bioactive compounds, including consistent yield and large-scale production in fermenters, obviating the need for collection of sponges from natural ecosystems (Zilinskas et al., 1995).

In this study, microbial symbionts were investigated in two Great Barrier Reef sponges, *Rhopaloeides odorabile* Thompson et al. (Dictyoceratida: Spongiidae) and a new species, termed here 'Very White Fan' (VWF) (see Bergquist et al., 1999, this volume). This is a first step towards ascertaining whether these symbionts are implicated in the production of important bioactive compounds. *Rhopaloeides odorabile*, common throughout the GBR, produces novel norsesterterpenes (rhopaloic acids) which exhibit potent cytotoxic activities (Ohta et al., 1996), and VWF contains the compound fanolide (P. Murphy, unpublished data), which retards the growth of several tumour cell lines.

MATERIALS AND METHODS

SPONGE COLLECTION AND BACTERIAL ISOLATION. Material examined in this study was collected using SCUBA (0-30m depth). Seasonal sampling for bacterial community studies was conducted over 12 months at Davies Reef (50 nautical miles off Townsville, Queensland, Australia, 18°49.6'S, 147°34.49'E). Immediately after collection, specimens were processed for bacterial isolation. Using aseptic technique, a $1cm^3$ section of sponge tissue was excised and surface-sterilised. Sponge tissue was homogenised in sterile artificial seawater (ASW) using a mortar and pestle. Serial dilutions (10^0, 10^{-1} and 10^{-2}) were prepared in ASW and plated onto several media for isolation of microbes.

Media for isolation of heterotrophic bacteria were used in this study. Difco Marine Agar 2216 as a non-selective marine medium, TCBS for enteropathogenic vibrios (Oxoid) and SBA, a selective medium for bacterial pathogens (Oxoid Columbia Blood Agar Base). BG-11 (Stanier et al., 1971) and MN + B12 (Waterbury & Stanier, 1978) were used for isolation of oxygenic phototrophs. Plates were incubated at 27°C for a period ranging between 2-4 weeks. Total culturable colony counts were obtained from Difco Marine Agar 2216 spread plates. Representatives of each morphotype were cultured from initial isolation and cryopreserved for further studies. Total bacterial counts were determined by fluorescent microscopic enumeration of cells stained with 4'6-diamidino-2-phenylindole (DAPI) as described by Porter & Feig (1980).

SCANNING AND TRANSMISSION ELECTRON MICROSCOPY. Sponge sections were prepared using a scalpel blade to cut 1-1.5mm thick sections of sponge tissue, ensuring that both the ectosome and choanosome were represented. Sections were fixed in 2.5% glutaraldehyde made in 0.1M sodium cacodylate buffer for 20hrs. Fixed samples were transferred into 0.1M sodium cacodylate and stored at 4°C until further processing. Sections of sponge tissue were placed in a 1% osmium tetroxide solution (prepared in 0.2M potassium phosphate buffer, pH 7.4) for 3.5hrs. Sections were removed and washed thoroughly in sterile distilled water, dehydrated in a graded ethanol series (15%, 35%, 55%, 75%, 85% and 95%), placed in embedding capsules and covered with Spurr's resin. Thin sections were cut and stained with 2% uranyl acetate followed by 0.2% lead citrate. Sections were mounted on 200 mesh copper TEM grids

TABLE 1. Type cultures used as control strains in biochemical analyses. Key: ACMM, Australian Collection of Marine Microorganisms; ATCC, American Type Culture Collection.

Collection number	Organism
ACMM 667	*Vibrio parahaemolyticus*
ACMM 668	*V. parahaemolyticus*
ACMM 89	*Vibrio alginolyticus*
ACMM 90	*V. parahaemolyticus*
ATCC 33809	*Vibrio fluvialis*
ATCC 33807	*V. fluvialis*
ATCC 7966	*Aeromonas hydrophila*
ATCC 35624	*Aeromonas* group 77
ATCC 33509	*Vibrio ordalii*

coated with carbon and Formvar. Samples were visualised following standard scanning and transmission electron microscopy techniques at James Cook University and University of Queensland.

BIOCHEMICAL TESTING OF BACTERIAL ISOLATES. The isolates were characterised by determining biochemical characteristics in 96-well microtitre-plates based on methods described by Hansen & Sorheim (1991). Several dye indicator tests were performed: Moller's arginine, lysine, ornithine, base; nitrate reduction, ONPG, indole, acetoin, tellurite, aesculin, alginate, acid-arabinose, arbutin, glucose, inositol, mannose, salicin, sorbitol, sucrose, and urea. The following tests were performed to determine the ability to utilise different carbon sources in assimilation broth: arabinose, cellobiose, galactose, glucose, mannose, melibiose, lactose, melizitose, sucrose, trehalose, xylose, ethanol, glycerol, propan-1-ol, sorbitol, gluconate, glucuronate, amygdalin, arbutin, citrulline, hydroxyproline, leucine, glucosamine, hydroxybutyrate, α-ketoglutarate, succinate, base (control), adenine, aminovalerate, N-acetyl-D-glucoseamine, ethanolamine, m-erythritol, D-fructose, D-galacturonate, glutarate, inositol, malonate, maltose and valerate. The assimilation broth contained (per litre) 0.015g of yeast extract, 1.0g of ammonium chloride, 0.075g of di-potassium hydrogen orthophosphate, 6.1g of Tris (hydroxymethyl) aminomethane and 15g of ASW salts (pH 7.5). After autoclaving, the carbon sources were filter sterilised and added aseptically to a final concentration of 8% (wt/vol.). Inoculations were performed by suspending colony material in ASW and inoculating 100µl of this suspension into each test. In addition, the following morphological characteristics were determined: colony morphology, gram stain and cell morphology, plate swarming, oxidation/fermentation (Leifson, 1963; Lemos et al., 1985), oxidase, catalase and growth at various salt concentrations (0%, 1%, 6%, 8%). In addition several antibiotic susceptibility tests (O/129 10 & 150µg, ampicillin 10µg & polymixin B 50iu) were performed along with growth on different media (Lecithinase, DNase, TCBS, SBA). Several American Type Culture Collection (ATCC) and Australian Collection of Marine Microorganism (ACMM) strains were included as controls (Table 1). Isolates were tentatively identified to either the genus or species level by comparing their phenotypic characteristics with those of type cultures and by comparing biochemical test results, carbohydrate utilisation patterns, and cell morphologies to those of species described in *Bergey's Manual of Systematic Bacteriology* (Holt, 1986) and *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons, 1974).

DATA ANALYSIS. The levels of relatedness of the bacteria were determined from the phenotypic data using Jaccard's similarity index (Zar, 1984).

$$S_j = a/(a + b + c)$$

where S_j =Jaccard's similarity coefficient, a = no. species in sample A and sample B (joint occurrences), b = no. species in sample B but not in sample A, c = no. species in sample A but not in sample B.

Phenograms were constructed by using unweighted pair group mean average (UPGMA) linkage (Sokal & Michener, 1958), Euclidean distances and the computer software package STATISTICA (StatSoft Inc., Tulsa, Oklahoma).

BACTERIAL IDENTIFICATION BY 16S RIBOSOMAL RNA (rRNA) ANALYSIS. Two microbial isolates, NW002 from *R. odorabile* and the microalga E30004315 were identified using a molecular approach, partial sequencing of the 16S rRNA gene fragments amplified from these isolates using the polymerase chain reaction (PCR). Total DNA was prepared from strains NW002 and E30004315 using a method modified from Ausubel et al. (1987). Oligonucleotide primers with specificity for eubacterial 16S rRNA genes [Forward primer 8-27:5'AGAGTTT GATCCTGGCTCAG -3' (modified from FD1) (Weisburg et al., 1991) and Reverse primer 1492:5'-GGTTACCTTGTTACGACTT-3' (Reysenbach et

al., 1992)] were used to amplify a 16S rRNA gene fragment from NW002. The cyanobacterial and plastid-specific 16S rRNA primers described by Nübel et al. (1997) were used for E30004315, since this isolate, although unialgal, may have been contaminated with low numbers of heterotrophic bacteria. PCR fragments were purified using the Microcon 30 system (Amicon, Beverly, MA), and sequenced using the PRISM Ready Reaction Kit (PE Applied BioSystems, Foster City, CA) and an ABI 310 sequencer (PE Applied BioSystems). Sequencing data were analysed by comparison to 16S rRNA genes in the Ribosomal Database Project (Maidak et al., 1999; Maidak et al., 1997) and the Genbank database, and aligned manually using the Phydit software (Chun, 1995).

Evolutionary trees were inferred using the neighbour-joining (Saitou & Nei, 1987), Fitch-Margoliash (Fitch & Margoliash, 1967) and maximum-parsimony (Kluge & Farris, 1969) algorithms in the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour- joining and Fitch-Margoliash methods were generated as described by Jukes & Cantor (1969). Tree topologies were evaluated by performing bootstrap analyses of the neighbour-joining data, based on 1000 re-samplings (Felsenstein, 1985).

Abbreviations: AIMS, Australian Institute of Marine Science; ASW, Artificial seawater; DAPI, Diamidino-phenylindole, 16S rRNA, 16S ribosomal ribonucleic acid; SBA, Sheep Blood Agar; TCBS, Thiosulphate Citrate Bile Salts Medium; VWF, Very White Fan.

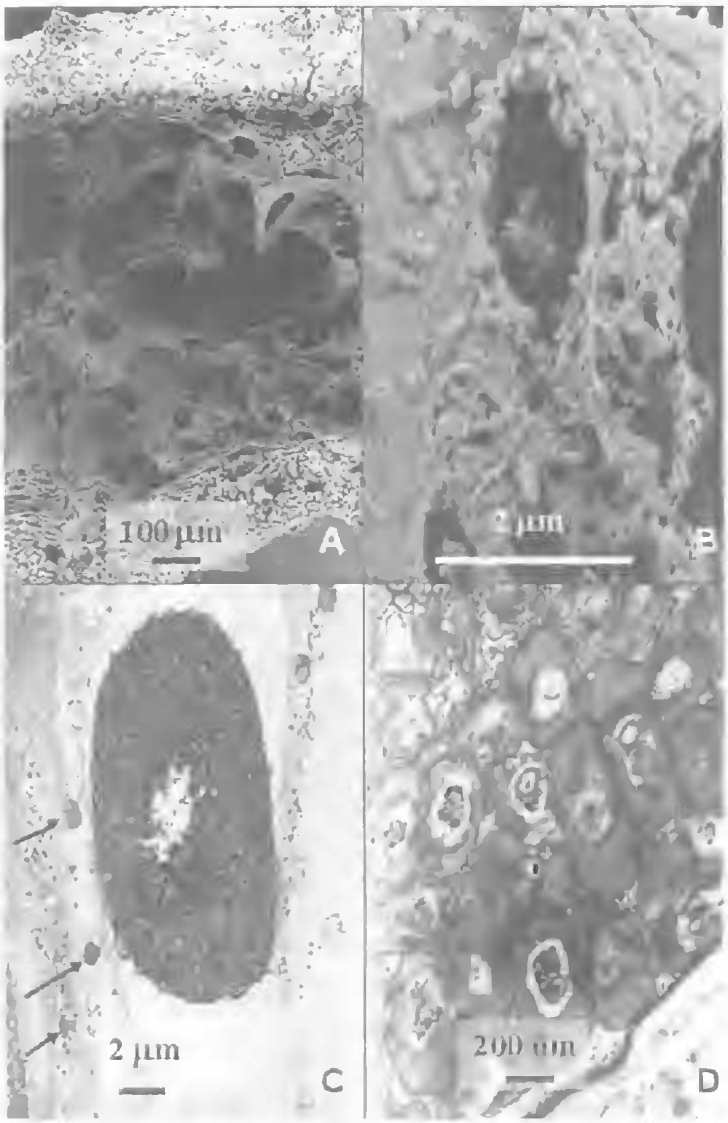

FIG 1. Electron micrographs of VWF sponge sections. A, Low magnification scanning electron micrograph of sponge section. B, High magnification of sponge section showing cells identified as putative cyanobacteria on morphological criteria. C-D, Low and high magnification, respectively transmission electron micrograph of sponge mesohyl section showing location of bacteria within 'bacteriocytes' and putative cyanobacteria, indicated by arrows.

RESULTS

ELECTRON MICROSCOPY. A large and complex bacterial community was shown by electron microscopy to be present within the sponge VWF (Fig. 1A-D). Bacteria closely associated with the sponge tissue, possibly embedded in a polysaccharide matrix, were presumed to be cyanobacteria based on morphological criteria (Fig. 1B), since these cells resemble filamentous cyanobacteria (e.g. genus

Oscillatoria). Cells presumed to be other eubacteria, based on the standard morphological criteria of size, shape and membrane structure, were also in close contact with the sponge tissue and contained in cellular organelles resembling the 'bacteriocytes' described by Vacelet & Donadey (1977) (Fig. 1C-D). Sand grains were observed which appeared to be incorporated into the sponge external structure (also reported by Bergquist, et al., 1999), possibly performing the function of increasing structural integrity (Shaw, 1927).

The bacterial community within *R. odorabile* was also large and appeared to be comprised of many different bacteria (Fig. 2). The bacteria appear to be dispersed throughout the sponge mesohyl and no bacteriocytes were evident. In contrast to VWF, cells resembling cyanobacteria were not seen.

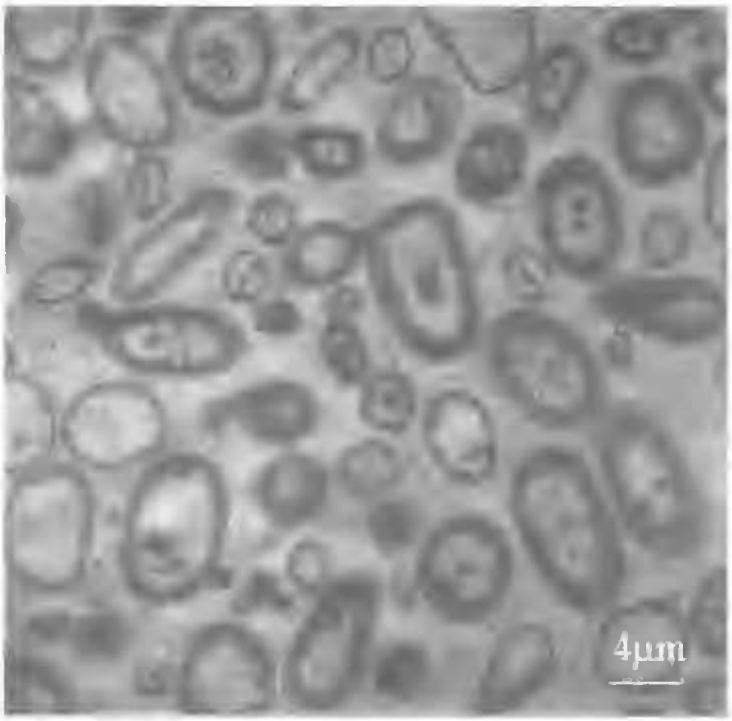

FIG. 2. Transmission electron micrograph showing the diversity of bacterial morphologies and the density of bacterial cells within the tissue of the sponge *R. odorabile*.

BACTERIAL ENUMERATION.
The average number of culturable bacteria from direct plate counts obtained from VWF was $3.6x10^4$/ml and the average total count observed from DAPI staining was $6.3x10^7$/ml. Only 0.06% of total bacteria were able to be recovered using traditional culture techniques. The range of total and culturable bacterial counts found in samples from eight individual VWF sponges are shown in Figure 3.

The average percentage of culturable bacteria from *R. odorabile* was only 0.1% with a range of 0.001-0.8%. The average percentage of bacteria able to be cultured from the water column was 0.23% with a range from 0.003-0.9%. Total and culturable bacterial counts found in samples from four individual *R. odorabile* sponges and the ambient seawater surrounding each sponge are shown in Figure 4.

BIOCHEMICAL CHARACTERISATION OF BACTERIAL ISOLATES. Morphological and biochemical data indicated that, at least as judged from the culturable fraction, the bacterial community within VWF differed from that present in the water column. Culture results from 15 VWF individuals from different locations revealed several similarities. Eight eubacteria, designated AB001 to AB008, were frequently observed as being part of the culturable bacterial community of VWF and were found to be present only in small numbers in samples from the water column.

A total of 220 isolates were isolated from VWF samples collected between June 1997-May 1998 from locations between Trunk Reef and Davies Reef, Great Barrier Reef. These isolates conformed to one of eight clusters (Fig. 5). Two clusters were Gram-positive with the remainder being Gram-negative. The Gram-positive bacteria were further sub-divided by means of cellular morphology. Approximately 40% of all bacteria (including strains AB004, AB007, and AB008) isolated from VWF clustered in phenons 6 and 7 (Fig. 5); these phenons contained the vibrio and aeromonad representatives, respectively, of the *Vibrionaceae* type strains used in this study. In addition, approximately 30% of the total bacterial community fell in a single phenon (phenon 8), which contained the strain AB005. Of the remaining three Gram-negative

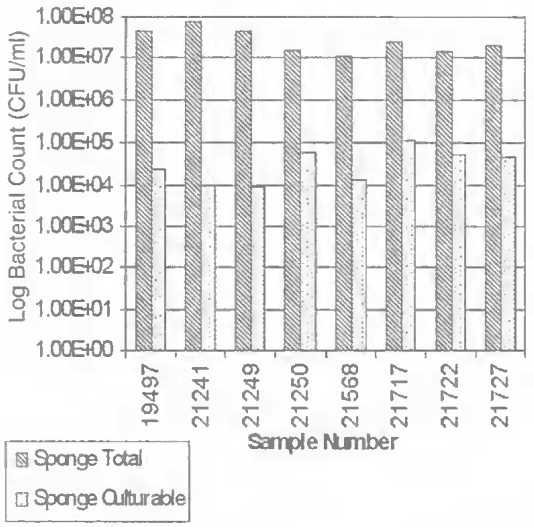

FIG. 3. Total and culturable bacterial counts from tissue of eight VWF individuals collected at Davies Reef, Great Barrier Reef.

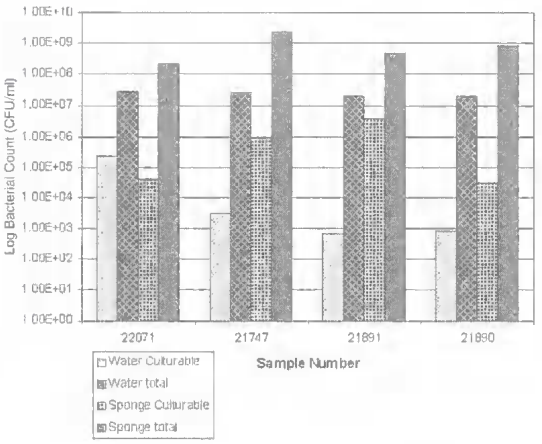

FIG. 4. Total and culturable bacterial counts from tissue of four *R. odorabile* individuals and seawater surrounding each individual collected at Davies Reef, Great Barrier Reef.

clusters, phenon 3 contained pigmented bacteria, phenon 4 included strains AB003 and AB004, and phenon 5 included strains AB001 and AB002. Strain AB001 appears closely related to NW001 from *R. odorabile*, with both strains being representatives of the alpha-Proteobacteria (data not shown).

From biochemical and morphological observations, it was apparent that the bacterial community within *R. odorabile* was quite distinct from the bacterial assemblages associated with the ambient water column. In general, both total and culturable counts from sponges exceeded counts from the corresponding water samples. The sponge microbiota was dominated by an organism designated NW001, whereas this isolate was completely absent in the surrounding water column. A small component of the microbial community was observed in both the sponge tissue and the ambient seawater.

A total of 223 isolates were collected from 40 *R. odorabile* samples collected between August 1997-May 1998. These isolates conformed to one of ten major clusters (Fig. 6). Two clusters (phenons 9 & 10) were Gram-positive and these were distinguished from each other on the basis of the oxidase reaction. Two of the Gram-negative clusters (phenons 1 & 2) were oxidase-negative and showed profiles linking them to the Enterobacteriaceae. One of the Gram-negative, oxidase-positive clusters (phenon 3) was catalase-negative and the remaining five clusters were catalase-positive and separated on the basis of carbon source utilisation. One of the five clusters contained an *Aeromonas* sp. type culture (phenon 6) and a second cluster contained the *Vibrio anguillarum* type culture (phenon 4). NW001 was a Gram-negative rod; oxidase, catalase, urease, VP and indole positive; utilised adenine dihydrogenase and had the ability to utilise glucose and gluconate as carbon sources. NW002 was a Gram-negative rod, oxidase, catalase, VP, indole and acid arabinose positive. It was urease--negative and did not utilise any of the tested carbon sources. Both NW001 and NW002 clustered within phenon 5.

MICROALGAL ISOLATES. In addition to the heterotrophic bacterial isolates, eight strains of oxygenic phototrophs were isolated from VWF and one of these strains, designated E30004315 was characterised by 16S rRNA sequencing (below). A single phototroph strain was isolated from *R. odorabile*. Phototroph strains were not characterised by biochemical testing because of the difficulty in identification of microalgae by this means; instead 16S rRNA sequencing was used as a method for identification of strain E30004315.

PHYLOGENETIC POSITIONS BASED ON 16S rRNA SEQUENCING. Phylogenetic relationships for the plastid of microalga E30004315 from VWF and heterotrophic bacterial strain NW002 from *R. odorabile* are shown in Figures 7 and 8,

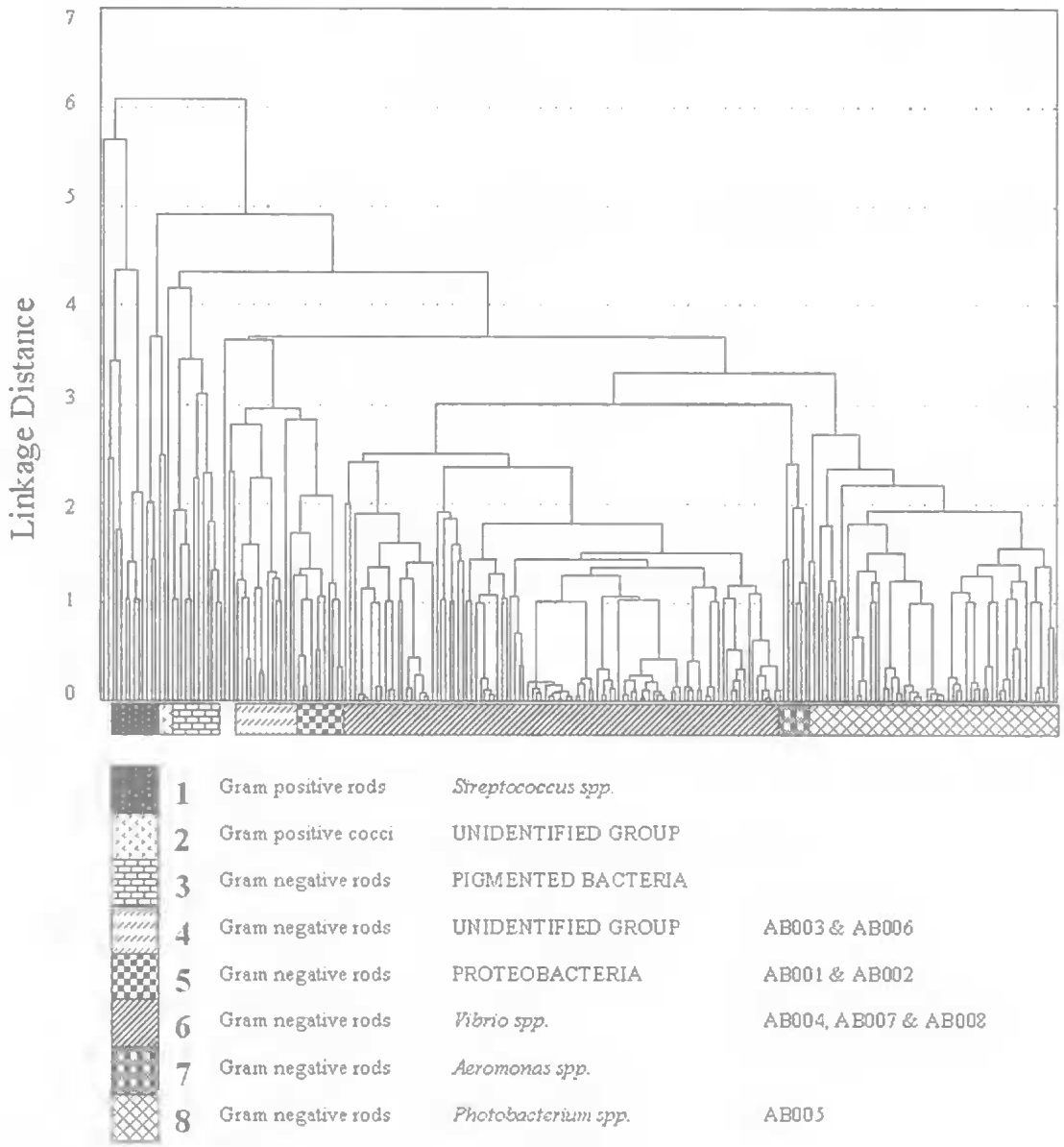

FIG. 5. Similarity dendrogram for isolates obtained from VWF.

respectively. The plastid from isolate E30004315 from VWF is closely related to plastids of other marine eukaryotic algae. NW002 is a *Pseudoalteromonas* sp.

DISCUSSION

This comprehensive biochemical and morphological analysis of bacteria isolated from two Great Barrier Reef sponges further emphasises the variability in microbiota associated with marine invertebrates. Several studies have documented diverse microbial communities associated with sponges (Vacelet, 1970, 1975; Vacelet & Donadey, 1977; Wilkinson, 1978a,b,c; Santavy, 1985; Willenz & Hartman, 1989; Santavy & Colwell, 1990; Santavy et al., 1990; Lopez et al., 1999). These communities are generally comprised of large numbers of heterotrophic bacteria that often occupy up to 60% of the sponge volume (Santavy, 1985; Wilkinson, 1978a,b).

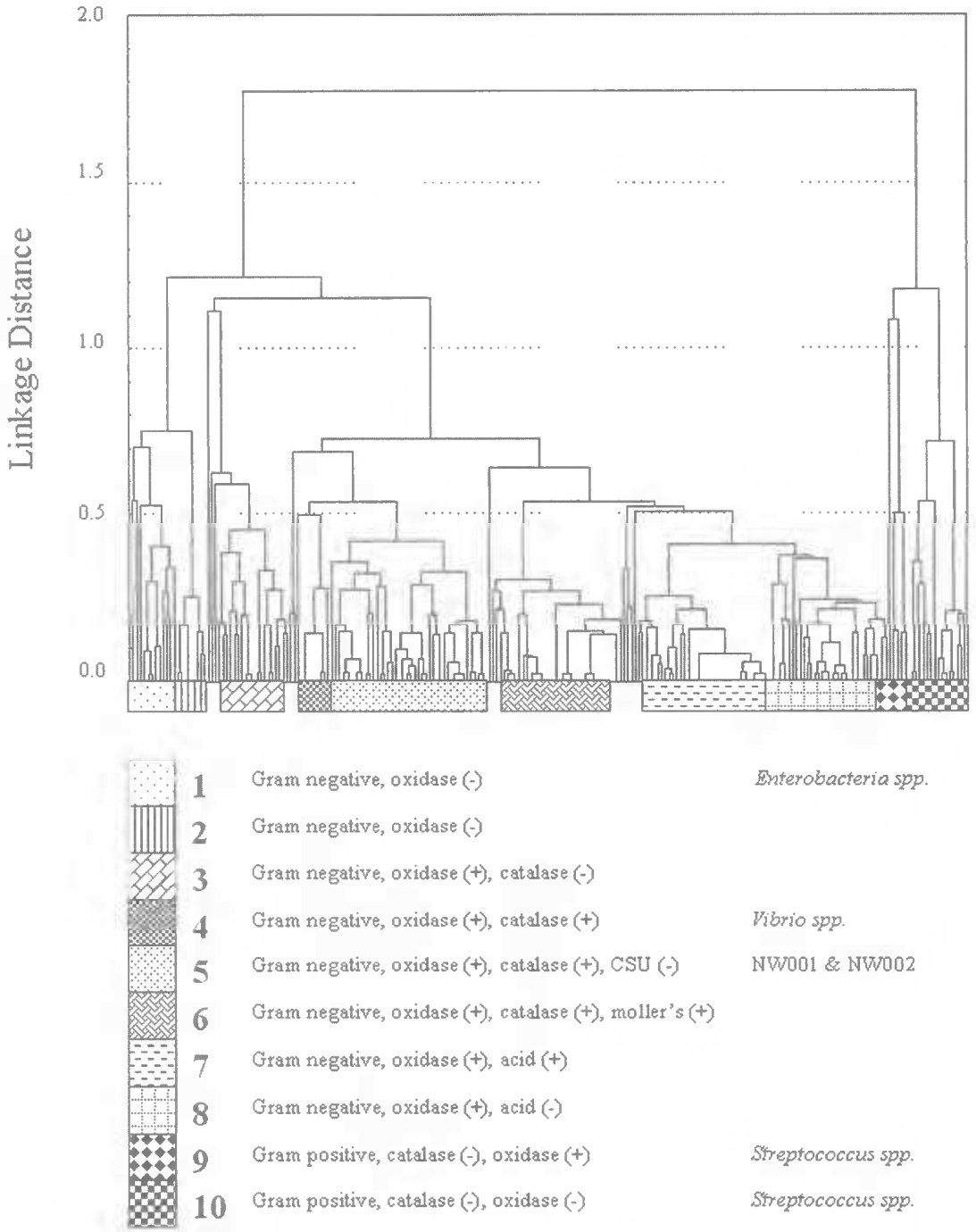

FIG. 6. Similarity dendrogram for isolates obtained from *R. odorabile*.

Phenotypic analysis of bacteria from the Caribbean sclerosponge, *Ceratoporella nicholsoni* revealed significant differences in sponge and seawater phenotypes (Santavy & Colwell, 1990). It is evident from the present study, that the sponges *Rhopaloeides odorabile* and VWF support taxonomically diverse microbial assemblages. High microbial diversity is not surprising when

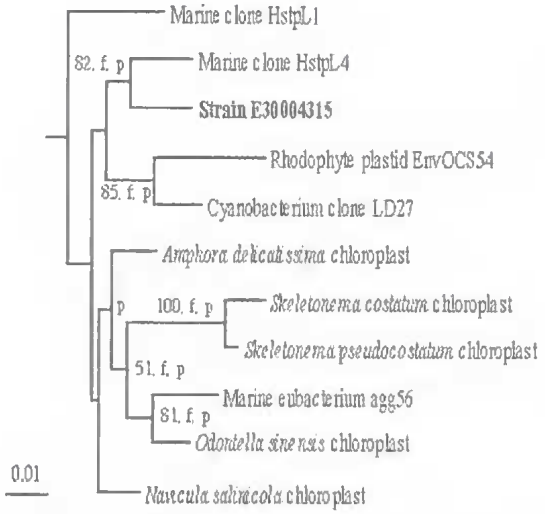

FIG. 7. Neighbour-joining tree for 631 bp of sequence obtained using cyanobacterial and plastid-specific primers from strain E30004315 isolated from VWF. Key: f and p indicate branches that were also found using the Fitch-Margoliash or maximum-parsimony methods, respectively. The numbers at the nodes are percentages (only values over 50% shown) indicating the level of bootstrap support, based on a neighbour-joining analysis of 1,000 re-sampled data sets. Scale bar represents 0.01 substitutions per nucleotide position.

considering that sponges derive their nutrition from filtering the ambient seawater. It has been demonstrated that marine sponges are capable of discriminating between food bacteria and bacterial symbionts. The mechanisms for this recognition are not clear but it has been postulated that sponge phagocytic cells do not recognise the capsule coating of symbionts (Wilkinson et al., 1984).

Eight predominant heterotrophic bacteria were evident in the culturable community isolated from VWF and these isolates were generally absent from the surrounding seawater samples. These culturable bacteria clustered in several phenons on biochemical analysis. The culturable community of VWF was more diverse than that observed in *R. odorabile* and showed greater fluctuations between individual sponges. One notable feature was the prevalence of cells resembling cyanobacteria within the VWF matrix, observed by microscopy. Also, eight strains of phototrophs were isolated from this sponge. It is postulated that VWF is a phototrophic sponge, deriving a component of its carbon budget from photosynthetic symbionts. Wilkinson (1992) has reported that many sponge phototrophs are morphologically flattened to increase surface area for interception of light. This is consistent with the structural morphology of VWF. In contrast, only a single phototroph was isolated from *R. odorabile* and cells with characteristic cyanobacterial morphology were not observed on microscopic examination of *R. odorabile* tissue.

The culturable bacterial community of *R. odorabile* was dominated by strain NW001, which comprised 74% of the total culturable bacterial community in this sponge but was consistently absent from the seawater samples. This is the first report of a single bacterium comprising such a high proportion of the culturable bacteria from a sponge. Previously, a specific bacterial symbiont was found in nine of ten sponges of two classes and seven orders, and a second symbiont was specific to the sponge *Verongia*, but only as one component of a large mixed bacterial community (Wilkinson et al., 1981). The relationship between NW001 and *R. odorabile* provides an ideal model system for investigating the relationship between this strain and its host sponge because this isolate is predominant and has a characteristic colony morphology which facilitates enumeration of strain NW001 based solely on colony morphology. Initial indications are that this relationship persists over spatial and temporal scales and is highly stable (work in progress). Although the relationship between NW002 and *R. odorabile* appeared less stable, NW002 was frequently the second most predominant culturable bacterium (after NW001) present in *R. odorabile*, and was present in the sponge tissue at much higher concentrations than detectable in the ambient water surrounding the sponges. Similarly, strains AB001-AB008 were consistently present in the sponge VWF at higher concentrations than detected in the surrounding seawater. The mechanism whereby the sponges acquire these symbionts is a topic of current research.

It appears as though the two sponges adopt different strategies for harboring their microbial communities. *Rhopaloeides odorabile* maintains the bacterial cells in the loose matrix of the mesohyl, whereas VWF appears to incorporate the cells into structures referred to as bacteriocytes. In VWF, the bacteria are also closely associated with the sand grains just below the cuticle. The reasons for these different approaches are uncertain but may relate to the structural composition of the sponge or the function of the bacteria within the sponge tissue. *Rhopaloeides odorabile* maintains

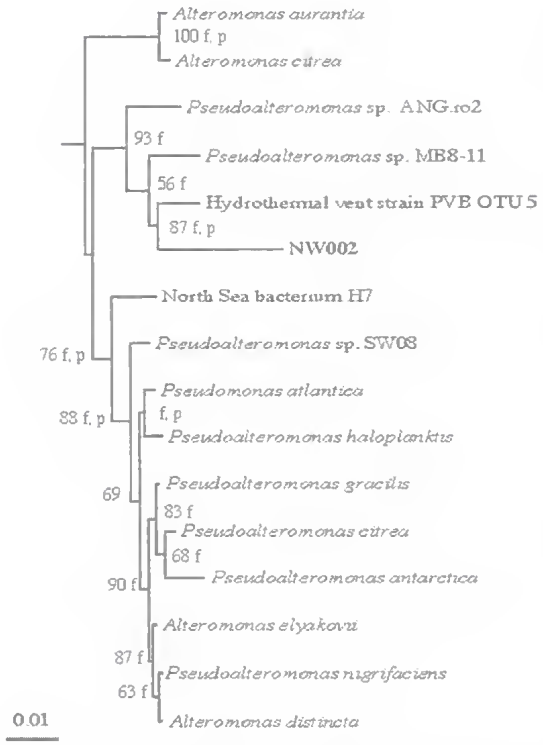

FIG. 8. Neighbour-joining tree for over 1,000 bp of sequence obtained using eubacterial-specific primers from strain NW002 isolated from *R. odorabile*. Key: f and p indicate branches that were also found using the Fitch-Margoliash or maximum-parsimony methods, respectively. The numbers at the nodes are percentages (only values over 50% shown) indicating the level of bootstrap support, based on a neighbour-joining analysis of 1,000 re-sampled data sets. Scale bar represents 0.01 substitutions per nucleotide position.

a bacterial community two orders of magnitude greater than that present in tissue of VWF.

Biochemical characterisation of all culturable isolates from VWF and *R. odorabile* was useful in clustering each of these assemblages into distinct phenons. In some cases, the presumptive identity of isolates could be deduced by comparison with type cultures which clustered in the same phenon. However, this approach must be used with caution because of the difficulty in identifying marine isolates based on criteria generally established for readily-culturable isolates of medical significance. In addition, many isolates scored negative against almost the entire biochemical profile, as is frequently the case with marine environmental isolates. Interestingly, two phenons from each sponge comprised Gram-positive bacteria, which made up approximately 10% of the total isolates in each case. Early studies of marine microbiology found that about 95% of marine isolates were Gram-negative (ZoBell, 1946) but recently 30% of the bacteria associated with a marine alga were found to be Gram-positive (Jensen & Fenical, 1995) and it is likely that the proportion of Gram-positive bacteria in most marine habitats has been underestimated (Jensen & Fenical, 1994). Gram-positive bacteria include actinomycetes, a group of particular importance in natural products discovery.

Because of the difficulties in accurately identifying marine bacteria by conventional biochemical characterisation, molecular techniques are the most appropriate for unequivocal identification of marine bacteria. A single isolate (NW002) from phenon 5 of the assemblage from *R. odorabile* was selected to demonstrate the utility of this approach and as a first step in the molecular identification of one isolate from each phenon. In addition, because of the difficulty in identification of marine microalgae by conventional techniques, phototroph isolate E30004315 was characterised by 16S rRNA sequence analysis of its plastid.

Sequence analysis of the plastid of microalgal strain E30004315 revealed that this plastid was most closely related to sequences of clones HstpL1 and HstpL4, cloned from a library of the uncultured microbes associated with the seagrass *Halophila stipulacea*, a ubiquitous seagrass from the subtidal zone of the Gulf of Elat (Weidner et al., 1996). Another close relationship was to clone OCS54, a plastid rRNA sequence from a natural phytoplankton population collected in the Pacific Ocean, off the mouth of Yaquina Bay, Oregon (Rappe et al., 1998). The identified microalgae with plastids clustering close to E30004315 fall in the genera *Odontella* and *Skeletonema* (Fig. 7). Microscopic examination of E30004315 revealed morphology consistent with identification as a microalga in the eukaryotic phytoplanton, with cigar-shaped cells about 10µm long.

Strain NW002 clearly belongs to the genus *Pseudoalteromonas*, a genus with many marine representatives, on the basis of the close phylogenetic relationship between this isolate and sequences of strains classified in the genus *Pseudoalteromonas*. The 16S rRNA sequence most closely related to that of NW002 was derived from a clone derived from a microbial mat at a hydrothermal vent site, the Loihi Seamount,

Hawaii and reported as an alteromonad (Moyer et al., 1995).

The percentage of culturable bacteria associated with these sponges was only 0.06% in VWF and 0.1% in *R. odorabile*. These percentages are considerably lower than the 3-11% culturable bacteria associated with the sclerosponge *Ceratoporella nicholsoni* (Santavy, et al., 1990), and may indicate that a high proportion of the bacteria associated with *R. odorabile* and VWF are obligate symbionts, requiring a close association with the sponge tissue to grow. These results illustrate the importance of molecular genetic techniques for total community analysis. Once more is known about the total microbial community associated with sponges, it may be possible to use this knowledge for rational selection of culture conditions appropriate for growth of additional, presently unculturable, strains. This study has resulted in an array of well-characterised microbes for natural products screening, in particular for important compounds known to be produced by these sponges. Compounds of potential pharmaceutical importance from *R. odorabile* include diterpenes (Kazlauskas et al., 1979) and rhopaloic acid A (Ohta, et al., 1996), although it is likely that these particular compounds are not of microbial origin (Thompson et al., 1987). VWF produces a potent antitumor compound fanolide. It is clear that marine sponges have the potential to be a major source of microbes for natural products screening programs.

ACKNOWLEDGEMENTS

We are grateful to Nicholas Gudkovs and Mark Crane from the Australian Animal Health Laboratory, Fish Diseases Laboratory, Geelong, for providing the original methodology and identification program used in biochemical testing. Adam Reynolds and Heather Windsor of James Cook University and Richard Webb at the Centre of Microscopy and Microanalysis of the University of Queensland are thanked for assistance with electron microscopy. Jacques Ravel is thanked for assistance in phylogenetic analyses.

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DISCOVERY AND SUSTAINABLE SUPPLY OF MARINE NATURAL PRODUCTS AS DRUGS, INDUSTRIAL COMPOUNDS AND AGRO-CHEMICALS: CHEMICAL ECOLOGY, GENETICS, AQUACULTURE AND CELL CULTURE. *Memoirs of the Queensland Museum 44: 76. 1999*:- Using chemical ecological clues, it is now possible to target habitats and eco-taxonomic groups of marine organisms to increase the likelihood of discovery of species which elicit natural compounds with chemotherapeutic or industrial application. Using the same clues, combined with Geographic Information System interrogation of the benthic geomorphology and oceanography associated with target species, it is possible to identify locations allowing recollection of species of interest. The information gained from both primary collections and focused recollections, provides the basis for hypothesis-driven experiments examining sustainable supply options for extracted target metabolites where synthesis is not practicable.

We describe recent results from an integrated multi-disciplinary programme designed to develop sustainable production options for a variety of marine natural products that have interesting biologieal activities. Three species of sponge from the genera *Lissodendoryx*, *Mycale* and *Latrunculia*, produce novel metabolites with anti-tumour activity. The natural abundance of each would not support a production industry based on wild harvest should their metabolites be required for drug production. Each has been successfully cultured in-sea demonstrating very good to excellent growth parameters. Each can be cultured with maintenance of target metabolite biosynthesis. In addressing the question of how to optimally produce target compounds, it has been necessary to examine a number of key biological issues pertaining to each species. These include genetic identity of populations supplying seed material, correlates with variable target metabolite biosynthesis in natural populations, origin of target metabolite biosynthesis (symbiont or sponge), and the efficacy of artificial production techniques (sea or land aquaculture or cell culture).

We conclude that the guess-work can now be taken out of artificial culture of sponges with a view to produce desirable natural products. It is possible to select for a high yielding culture stock and provide techniques to enhance biosynthesis or target metabolites. ❐ *Porifera, marine natural products, aquaculture, genetics, cell culture.*

C.N. Battershill, M.J. Page, A.R. Duckworth (email: a.duckworth@niwa.cri.nz) & K.A. Miller**, National Institute of Water and Atmospheric Research, P.O.Box 14-901, Kilbirnie, Wellington, New Zealand; P.R. Bergquist, School of Biological Sciences, Auckland University, Private Bag, Auckland, New Zealand; J.W. Blunt, M.H.G. Munro, Chemistry Department, University of Canterbury, Private Bag, Christchurch, New Zealand; P.T. Northcote, Chemistry Department, Victoria University, Private Bag, Wellington, New Zealand; D.J. Newman, Natural Products Branch, Bldng 1052, Rm109, Box B, National Cancer Institute, Frederick, MD 21702-1201, USA; S.A. Pomponi, Harbor Branch Oceanographic Institute, 5600 Old Dixie Highway, Fort Pierce, FL 34946, USA; Present addresses: *Australian Institute of Marine Science, PMB 3, Townsville MC, Qld., 4810, Australia; **University of Wollongong, Northfields Ave, Wollongong, NSW 2500, Australia; 1 June 1998.*

CHARACTERIZATION OF CALCIUM-BINDING MATRIX PROTEINS FROM DISTINCT CORALLINE DEMOSPONGES. *Memoirs of the Queensland Museum 44: 76. 1999*:- Calcified sponges played an important role as reef building organisms during different geological time periods. Living relatives of this group investigated here, *Spirastrella (Acanthochaetetes) wellsi*, *Astrosclera willeyana* and *Vaceletia n. sp.*, can be found in cryptic niches of indopacific coral reefs. The first known relatives of some of these sponges are known since the upper permian.The mode of biomineralization of the examined species seems to be extremely conservative, since they are phylogenetically very old and exhibit merely minor alterations in their calcareous skeletons. Each of the three species exhibits a unique type of basal skeleton with its own specific modifications of carbonate crystals. Each species was shown to have a specific array of calcium-binding macromolecules enclosed within its intraskeletal matrix. The proteins are separated by SDS polyacrylamide gel electrophoresis. A single protein was detected in *S. wellsi*, two proteins in *A. willeyana*, and four proteins in *Vaceletia n. sp.*. All proteins were characterized by their molecular weight and isoelectric point. The soluble matrix constituents of each species were tested for their potential to decrease precipitation of calcium and strontium carbonate, respectively, in a saturated solution. The findings strongly suggest that these soluble proteins function as the template for skeletal formation and are responsible for determining the particular type of calcium carbonate polymorphs. ❐ *Porifera, biomineralization, organic matrix, calcium-binding proteins, calcite, aragonite.*

Matthias Bergbauer (email: berggaji@mailszrz.zrz.tu-berlin.de), Robert Lange, Ulrich Szewzyk, FG Ökologie der Mikroorganismen, Franklinstr. 29, OE5, Technische Universität Berlin, 10587 Berlin, Germany; Joachim Reitner, Inst. & Museum für Geologie & Paläontologie, Goldschmidstr. 3, Universität Göttingen, 37077 Göttingen, Germany; 1 June 1998